now has been found to disappear in growing Brucella cultures in the presence of antisera prepared against Brucella DNA but not in the presence of normal rabbit serum. It would be most desirable to extend the study of the influence of these antisera to include a variety of biological systems, such as bacterial transformation mediated by highly polymerized DNA. Such studies are planned for the near future.

The present information fails to reveal whether the formation of specific antibodies requires both protein and DNA or involves only the DNA molecule (and some of its breakdown products). However, from a genetic, biological and analytical standpoint, it would appear that the answer to this question may be less critical than the indication that DNA (and some of its breakdown products) may contribute to the specificity of an antigen. It must be determined in future studies to what extent the protein present in phenol-extracted bacterial DNA contributes to the formation of what appear to be DNA-specific antibodies, and whether such protein, if required, must be associated with the DNA in a uniquely stable fashion. Such nucleoprotein stability is indicated by some of the characteristics of the material here used.[8] Heidelberger and co-workers[16] previously have called attention to a bacterial nucleoprotein of unusual stability, which was antigenic and dissociated only by treatment with alkali.

The results here reported would seem to furnish procedures applicable to further studies on quantitative aspects of problems associated with DNA antigenicity. In particular, quantitative precipitin reactions with polymerized and DNAase-treated DNA, extracted by different procedures and from different species, should be studied with homologous and heterologous antisera. If immunochemical techniques can be used reliably to detect differences between DNAs of different species on the basis of the dependence of cross-reactivity upon degree of polymerization, it may become possible to obtain additional information regarding the chemical nature of DNA. Also, if future studies confirm the present evidence for antigenic specificity of DNA, it may become possible to reinvestigate the feasibility of affecting genetic processes that are assumed to be controlled by DNA. However, it is possible that specific alterations by antisera of intracellular "information centers" for hereditary traits may continue to prove difficult due to permeability barriers.

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[CONTRIBUTION FROM THE LILLY RESEARCH LABORATORIES]

The Isolation of a Second Antibiotic from *Streptomyces hygroscopicus*

BY ROBERT L. MANN AND W. W. BROMER

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S. hygroscopicus has been shown to produce a second antibiotic substance, hygromycin B. Isolation of hygromycin B was accomplished by chromatography on a cation-exchange resin, carbon adsorption and countercurrent distribution. Properties and partial characterization of the new antibiotic have been described.

The isolation of hygromycin from *S. hygroscopicus* and the determination of its structure have been reported.[1-3] In the early stages of the purification of hygromycin, paper chromatographic evidence was obtained that more than one antibiotic was produced by the organism. This paper is concerned with the isolation and partial characterization of a second antibiotic substance, hygromycin B. This new antibiotic is primarily of importance because of its activity against helminths, including ascarids in swine.[4]

Broths suitable for the isolation of hygromycin B were fermented according to methods described for the production of hygromycin.[1] Antibiotic potency was determined with *Bacillus subtilis* using a 16 hr. paper disc diffusion assay. A crude concentrate of hygromycin B was obtained by removing the antibiotic from the fermentation broth with a cation-exchange resin (Amberlite IRC-50 in the Na^+ cycle) and eluting with 0.1 *N* hydrochloric acid. Further purification was achieved by adjusting the resin eluate to *p*H 10.5 and treating with carbon (Norite S.G.) to adsorb the hygromycin B. The carbon was removed by filtration, washed with water, and the antibiotic was eluted with a solvent composed of 1 part of concentrated ammonium hydroxide, 3 parts of water and 6 parts of acetone. The eluate was concentrated to about one-tenth of the original volume and the hygromycin B was precipitated with acetone. The gummy precipitate was dissolved in methanol. When ether was added, a white flocculent precipitate of hygromycin B separated. This preparation was about 80% pure. Final purification was accomplished by adsorption of the antibiotic on a cation-exchange resin (Amberlite IRC-50 in the Li^+ cycle) followed by elution with 20% ammonium hydroxide.

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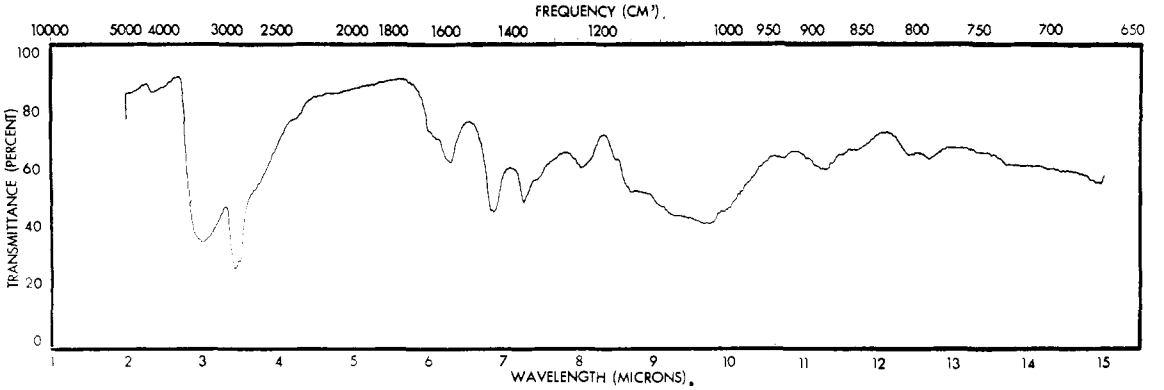

Fig. 1.— Infrared absorption spectrum of hygromycin B as a mull in mineral oil.

Homogeneity of the purified antibiotic was demonstrated by countercurrent distribution through 110 transfers using a solvent system composed of 1 part of methanol, 25 parts of water and 25 parts of 2-butanol. Both solids content and microbiological activity were in good agreement with a theoretical distribution curve.

Properties of Hygromycin B.—Hygromycin B is a polyhydroxy base melting over a wide range in the neighborhood of 180°. It is very soluble in water and methanol, but it is essentially insoluble in the less polar solvents.

Electrometric titration in water showed two titrable groups: one with pK_a' 7.1 and the other with pK_a' 8.8. Only a minor shift in either pK_a' value was observed when the antibiotic was titrated in 66% dimethylformamide, indicating that both groups are basic.[5] The apparent molecular weight determined from titration data is 398 ± 8.

Hygromycin B shows no ultraviolet absorption. Its infrared spectrum (Fig. 1), obtained as a mull in mineral oil, has no outstanding features other than strong absorption in the 3 μ region, characteristic of –OH and –NH.

The antibiotic reacts positively in the anthrone and Molisch tests. It does not reduce Benedict or Fehling solutions. Although hygromycin B was obtained only as an amorphous powder, a crystalline *p*-(*p*-hydroxyphenylazo)-benzenesulfonic acid salt has been prepared. Analyses of this crystalline salt and of the amorphous antibiotic indicate that the formula of hygromycin B is $C_{15}H_{28}N_2O_{9-10}$. Analyses were consistent with the presence of one N-methyl group; no O-methyl or C-methyl groups were found.

The antimicrobial spectrum of hygromycin B includes both gram-positive and gram-negative organisms as well as a number of fungi. However, the quantities required to prevent growth of sensitive organisms *in vitro* are large compared with those of most therapeutically useful antibiotics. Inhibitory concentrations range from 6.2 mcg. per ml. to 100 mcg. per ml. as determined by the usual agar dilution test. Perhaps the most unique biological activity of hygromycin B is its antiparasitic effect in swine.[4]

Acknowledgments.—The authors are grateful to Mr. Paul W. Landis for physicochemical data; to Messrs. W. L. Brown, H. L. Hunter, G. M. Maciak and Miss Gloria Beckmann for microanalyses; to Messrs. E. A. Presti and R. P. Perkins for technical assistance; and to Dr. C. W. Pettinga for helpful advice.

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Experimental

Chromatography on Amberlite IRC-50 (Na^+ Cycle).[6]—Thirty-six l. of filtered fermentation broth containing 86 g. of hygromycin B was passed over a 5 × 57 cm. IRC-50 (Na^+ cycle) column at a rate of 25 ml. per min. The column was then washed with 11.5 l. of water to remove most of the colored contaminants. Hygromycin B was eluted from the resin with 36 l. of 0.1 *N* hydrochloric acid. The eluate contained the equivalent of 82.5 g. of crude antibiotic. At this stage the preparation was about 50% pure.

Adsorption on Carbon.—The eluate (36 l.) from the IRC-50 column was adjusted to *p*H 10.5. Norite S. G.[7] (3.6 kg.) was added and the mixture was stirred one hr. and filtered. The carbon cake was washed twice with 10-l. portions of water. Hygromycin B was eluted from the carbon by stirring 30 min. with two 18-l. portions of a concentrated ammonium hydroxide:water:acetone (1:3:6) solvent system. The eluate was concentrated to 2 l. and was added slowly, with stirring, to 10 l. of acetone. The gummy precipitate which formed was dissolved in 1 l. of methanol and was poured, with stirring, into 10 l. of ether. The light-colored precipitate of hygromycin B which separated was removed by filtration, washed three times with ether and dried. The yield was 75 g. of 80% pure hygromycin B.

Chromatography on Amberlite IRC-50 (Li^+ Cycle).—A stirred aqueous suspension of IRC-50 was treated with solid lithium hydroxide until the *p*H reached 8.0. The *p*H was then adjusted to 7.0 with concentrated phosphoric acid, and 250 ml. of the prepared resin was packed in a 1.6 × 29 cm. column. Five g. of hygromycin B from the carbon purification step was dissolved in 50 ml. of water and was passed over the resin. The column was washed with water to remove color, and the antibiotic was then eluted with 29% ammonium hydroxide. The active fractions were combined and concentrated to dryness *in vacuo*. The residue was dissolved in 10 ml. of methanol and added dropwise to 500 ml. of ether. The resulting precipitate was washed three times with ether and dried. The yield was 3.2 g. of pure hygromycin B.

Countercurrent Distribution.—The solvent system was prepared by equilibrating a mixture of 1.5 l. of 2-butanol, 1.5 l. of water and 60 ml. of methanol. A 110 transfer countercurrent distribution was performed in an all-glass apparatus having a capacity of 10 ml. per phase in each tube. Ten ml. of the bottom phase was used to dissolve 500 mg. of hygromycin B obtained from the lithium hydroxide-treated resin. Upon completion of the distribution, samples were withdrawn from each tube for microbiological assay and for determination of solids. The distribution coefficient is 0.13. The contents of tubes 8 through 16

(6) Amberlite IRC-50 was obtained from Rohm and Haas Co., Philadelphia.

(7) Norite S.G. was obtained from R. W. Greeff and Co., Inc., Chicago.

were combined and concentrated to dryness *in vacuo*. The residue was dissolved in 2 ml. of methanol and added dropwise with stirring to 50 ml. of ether. The resulting precipitate was washed three times with ether and dried.

Anal. Calcd. for $C_{15}H_{28}N_2O_{10}$: C, 45.45; H, 7.12; N, 7.07; N-methyl, 3.79. Found: C, 45.77, 45.27; H, 7.63, 7.55; N, 6.92, 7.13; N-methyl, 3.93.

p-(p-Hydroxyphenylazo)-benzenesulfonic Acid Salt of Hygromycin B.—To 100 mg. of hygromycin B in 0.5 ml. of water was added 200 mg. of *p*-(*p*-hydroxyphenylazo)-benzenesulfonic acid in 2 ml. of water. The solution was cooled to 5°. After one hour the precipitate that formed was removed by filtration and was recrystallized three times from water. The yield was 124 mg. The salt gradually decomposed above 210°. An unexplained inconsistency in the carbon analyses of a number of salt preparations prevents the unequivocal assignment of a formula. However, one of the following two formulas appears to be correct: $C_{39}H_{48}N_6S_2O_{18}$ or $C_{39}H_{48}N_6S_2O_{17}$. Analytical values for two preparations are shown.

Anal. Calcd. for $C_{39}H_{48}N_6S_2O_{18}$: C, 49.15; H, 5.08; N, 8.82; S, 6.73. Calcd. for $C_{39}H_{48}N_6S_2O_{17}$: C, 49.99; H, 5.16; N, 8.97; S, 6.84. Found: C, 49.36, 50.13; H, 4.60, 5.06; N, 8.20, 8.69; S, 6.48, 6.67.

INDIANAPOLIS 6, INDIANA

[CONTRIBUTION NO. 2276 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

The α-Chymotrypsin-catalyzed Hydrolysis of α-N-Benzoyl-β-(4-pyridyl-1-oxide)-L-alanine Methyl Ester and of α-N-(Nicotinyl-1-oxide)-L-phenylalanine Methyl Ester[1]

BY ROBERT L. BIXLER AND CARL NIEMANN[2]

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Consideration of the consequences of multifunctionality of the catalytically active site of α-chymotrypsin and of a class of its representative specific substrates led to the conclusion that under comparable conditions the rate of the α-chymotrypsin-catalyzed hydrolysis of α-N-(nicotinyl-1-oxide)-L-phenylalanine methyl ester should be substantially greater than that of α-N-benzoyl-β-(4-pyridyl-1-oxide)-L-alanine methyl ester. This prediction has been verified by the observation that the relative rate of hydrolysis of the former specific substrate is *ca.* 200 times greater than that of the latter.

If it is assumed that the catalytically active site of α-chymotrypsin and its representative specific substrates, derived from α-amino acids, are multifunctional,[3-8] we may represent the specific substrate by the notation $R_1CHR_2R_3$, where R_1 is the acylamino moiety, R_2 the α-amino acid side chain and R_3 the group capable of undergoing reaction, and the quasi complementary loci of the catalytically active site by the symbols, ρ_1, ρ_2 and ρ_3. With such a representation and when the nature of R_3 is invariant, we may postulate that with all other reaction parameters held constant the rate of formation of reaction products will be dependent upon the frequency with which interactions of the type R_1-ρ_1, R_2-ρ_2 and R_3-ρ_3 are consummated, recognizing that the occurrence of such events will be dependent upon the degree to which $R_1 \neq R_2 \neq R_3$ and the relative magnitude of the various R-ρ interactions.[4-8] The correlative postulate is that R-ρ interactions other than those of the R_1-ρ_1, R_2-ρ_2 and R_3-ρ_3 type will be essentially non-productive and competitive.

When the R_2-ρ_2 interaction is dominant and when $R_1 \neq R_2 \neq R_3$, it would be expected that the probability of productive modes of combination would be greater than when $R_1 = R_2 \neq R_3$, because in the latter case interactions of the type R_2-ρ_1 and R_1-ρ_2 would tend to occur with greater frequency and thus increase the probability of unproductive and competitive modes of combination. A more extreme case would arise when fragments of the groups $R_1 \neq R_2$ are interchanged to produce a pair of specific substrates of the type $R'CONHCH(CH_2R'')CR_3$ and $R''CONHCH(CH_2R')CR_3$, where R_3 is invariant and where the nature of R′ and R″ are such as to minimize steric differences and to maximize differences in binding energies with respect to interaction with the ρ_1 and ρ_2 loci so that the order of affinity for the ρ_2 locus is R″ > R′. From the argument given above it would be expected that in the presence of α-chymotrypsin the first specific substrate would be hydrolyzed at a substantially faster rate than the second with all other factors held constant.

Earlier studies[9,10] had shown that the interaction of the catalytically active site of α-chymotrypsin with benzamide was considerably more favorable than its interaction with nicotinamide, presumably because of a greater extent of solvation of the latter species.[11] This knowledge coupled with that with respect to the so-called side chain specificity of α-chymotrypsin[3] prompted the intended comparison of the behavior of α-N-nicotinyl-L-phenylalanine methyl ester with that of α-N-benzoyl-β-(4-pyridyl)-L-alanine methyl ester[12] in the presence of α-chymotrypsin with the expectation that the former specific substrate would be hydrolyzed more rapidly than the latter.

When it was found that β-(4-pyridyl)-DL-alanine was not very soluble in water,[13] it was decided

(1) Supported in part by a grant from the National Institutes of Health, U. S. Public Health Service.

(2) To whom inquiries regarding this article should be sent.

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(12) This pair was chosen rather than the pair α-N-isonicotinyl-L-phenylalanine methyl ester and α-N-benzoyl-β-(4-pyridyl)-L-alanine methyl ester, arising from the simple interchange of the groups R′ and R″, because of a desire to maintain more nearly constant distances between the pyridine nitrogen atoms and the asymmetric carbon atoms of the members of the pair.

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